“A straightforward synthesis of 5'-peptide oligonucleotide conjugates using N^α-Fmoc-protected amino acids.” Ocampo, S.M., Albericio, F., Fernández, I., Vilaseca, M., Eritja, R. *Org. Lett.*, 7(20), 4349-4352 (2005).
 PMID: 16178530, doi: 10.1021/ol0514698

A straightforward synthesis of 5'-peptide oligonucleotide conjugates using N^α-Fmoc- protected amino acids

*Sandra M. Ocampo,[†] Fernando Albericio,[‡] Irene Fernández,[‡]
 Marta Vilaseca[‡] and Ramon Eritja^{†, *}*

Institut de Biologia Molecular de Barcelona (IBMB). C.S.I.C., Jordi Girona 18-26, E-08034, Institut de Recerca Biomèdica de Barcelona, Parc Científic de Barcelona, Josep Samitier 1-5, E-08028 and Departament de Química Orgànica, Facultat de Química, Universitat de Barcelona, Martí i Franqués 1-11. E-08028 Barcelona. Spain.

Abstract

5'-Peptide oligonucleotide conjugates were prepared stepwise on a single support using N^α-Fmoc- protected amino acids and unprotected phosphate groups. The method uses commercially available reagents and is successful with most natural amino acids. The simplicity of the method may encourage researchers to prepare new oligonucleotide-peptide conjugates with novel properties.

The use of synthetic oligonucleotides to control gene expression has triggered the search for new oligonucleotide derivatives to improve their therapeutic potential.¹ Oligonucleotide-peptide conjugates are chimeric molecules consisting of oligonucleotides covalently linked to peptides. As a result, synthetic oligonucleotides acquire some of the biological and/or biophysical properties of peptides. Thus, linking peptides to oligonucleotides may have beneficial effects such as: (a) facilitating oligonucleotide transport through cell membranes², (b) increased stability to exonucleases³, (c) improved binding to complementary sequences⁴, and (d) greater rate of hybridization⁵.

Two strategies can be followed to synthesize oligonucleotide-peptide conjugates.⁴ In the post-synthetic conjugation approach, the two moieties are prepared independently and specific groups (such as thiols and maleimido) are specifically incorporated to link both molecules.⁶ In the stepwise approach, oligonucleotide-peptide conjugates are prepared by stepwise addition of amino acids and nucleobases in solid phase on the same solid support.⁷ In this case, the problem is the incompatibility of the standard schemes of protection for peptides and oligonucleotides. For example, at the end of the solid-phase peptide synthesis, a treatment with acid is usually required, which can provoke partial depurination of DNA. In the synthesis of oligonucleotide 3'-peptides, this problem could be averted by using N^α-tert-butoxycarbonyl (Boc)-protected amino acids with base labile groups for the protection of side chains, a base-labile linker and standard phosphoramidites,^{7a-e} or by using N^α-9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids.^{7f-h} But these methods could not be applied for the incorporation of peptides at the 5' end or in the middle of the oligonucleotide sequence, as required for some applications such as gene delivery.⁸

Recently, a method for the solid-phase synthesis of oligonucleotide-5'-peptide conjugates has been described.⁹ It is based on the use of a protecting group [2-(biphenyl-4-yl)propan-2-yloxycarbonyl, Bpoc] for the α-amino group of the amino acids that can be removed in mild acid conditions suitable for DNA. Similarly, the monomethoxytrityl (MMT) group has been used for the synthesis of DNA/PNA chimeras.¹⁰

In this paper we describe the use of common Fmoc-amino acids for the stepwise synthesis of oligonucleotide-5'-peptide conjugates. Although the Fmoc-removal conditions are not orthogonal to the base-labile cyanoethyl protection of the DNA phosphotriester moieties, we found that the presence of unprotected phosphate groups did not hinder the assembly of small peptide sequences on oligonucleotide supports.

NPPLPPGPC is a proline-rich peptide that has recently been prepared in our laboratory for NMR structural studies using standard Fmoc chemistry. We chose this as a model to test the methodology because it does not contain trifunctional amino acids. The self-complementary octanucleotide sequence (5'CCAATTGG3') was prepared on controlled pore glass (CPG), polystyrene (LV200) and polystyrene-polyethylenglycol (PS-PEG) supports using standard 2-cyanoethyl phosphoramidite protocols. After the assembly of the oligonucleotide sequence, an amino group was added to the 5'-end using the N-6-MMT-aminoethyl phosphoramidite (Scheme 1). Aliquots of the 5'-amino support carrying the 5' amino oligonucleotide were placed in a syringe and the tripeptide PGP was assembled using standard Fmoc-chemistry (Scheme 1). It was also assembled using N^α-trityl-protected amino acids.¹¹ Unfortunately, during the latter assembly reactivity of the carboxyl functions was poor due to steric hindrance of the trityl group and severe truncation by acetylation was observed. Acetylation (from capping reagents) together with isobutyrylation and benzylation of the peptide sequence was described by Zaramella et al.⁹ as one of more severe side reactions occurring during the assembly of peptide at the 5'-end of the oligonucleotides. Surprisingly, the peptide sequence assembled using N^α-Fmoc-protected amino acids showed only mild truncation by acetylation at the 5'-aminooligonucleotide site even after repetitive capping with acetic anhydride after each amino acid addition (see Figure S1). We believe that the piperidine treatment applied during the removal of the Fmoc group eliminates the "reactive" acetyl groups in the support and the assembly of the peptide sequences is thus more efficient. We also found that polystyrene (LV200) and CPG supports gave good results, the former being slightly better. Unfortunately, the oligonucleotide-peptide conjugate could not be obtained on the PS-PEG support.

Scheme 1. Solid-phase synthesis of 5'-peptide oligonucleotide conjugates.

Next we prepared the heptapeptide sequence as well as related tri- and tetrapeptide sequences with trifunctional amino acids on CPG supports carrying 5'-amino oligonucleotides (see Table 1). Whenever possible, protecting groups labile to mild acidic conditions (such as 1% trifluoroacetic acid) were used to protect side chains of N^α-Fmoc-protected amino acids.

Amino acids with a carboxylic acid side-chain (Asp, Glu) were protected as 2-phenylisopropyl (PhiPr) esters. First, the hexanucleotide NPD(PhiPr)PC-5'CAATTG3' was assembled. The support was then treated with 3% trichloroacetic acid (TCA) in CH₂Cl₂ for 10 min to remove the PhiPr group, and then with ammonia. The desired oligonucleotide-peptide conjugate was present as the major component of the crude. Unfortunately, ammonia removed the PhiPr group if TCA treatment was omitted. It was important ensure that the PhiPr group was completely removed by the TCA treatment since hydrolysis by ammonia may lead to the corresponding amide instead of carboxylic acid. The amide peptide is difficult to detect. Using a model peptide (PheGluPro-amide) prepared on polystyrene we demonstrate that the PhiPr group is completely removed by 3% TCA giving glutamic acid (see supporting information). Therefore, after ammonia deprotection the desired oligonucleotide-peptide conjugates carrying glutamic acid are obtained.

Unprotected Fmoc-Asn and Fmoc-Gln can be used for the synthesis of peptides, although these amino acids are frequently protected with the trityl (Trt) group. We attempted to use both unprotected and Trt-protected glutamine (entries 6 and 9, Table 1) but we observed that the trityl group of glutamine was not removed by 3% TCA (entry 6, Table 1). Stronger acidic conditions will be needed to remove the Trt group which are not appropriate for DNA. In contrast, the desired tetrapeptide (entry 9, Table 1) was obtained using unprotected Fmoc-glutamine.

Two side-chain derivatives of lysine were tested successfully. The derivative of lysine carrying the trifluoroacetyl (Tfa) group is already used for the synthesis of

oligonucleotide-3'-peptide conjugates because it is removed in ammonia.⁷ Sequence NPKPC-5'CCAATTGG3' (entry 4, Table 1) was obtained in good yields using Fmoc-Lys(Tfa)-OH. Furthermore, the derivative of lysine carrying the acid labile MMT group was appropriate for the preparation of the oligonucleotide carrying the tetrapeptide NPKEPC-5'CCAATTGG3' (entry 9, Table 1). This result was expected because the MMT group also protects the 6-aminoethyl moiety. The nuclear localization signal (NLS) peptide AKKKKLDP was also prepared using Fmoc-Lys(Tfa)-OH and Fmoc-Asp(PhiPr)-OH (entry 14, Table 1).

Next, four derivatives of histidine were tested. The Trt derivative described previously for the preparation of oligonucleotide-3'peptide conjugates¹² was too stable because, like Trt in Gln, it was not removed by 3% TCA (entry 7, Table 1). In contrast, the Boc-protected derivative was removed by 3% TCA (entry 10, Table 1). The base labile Fmoc and the tosyl derivatives were also suitable for the preparation of conjugates carrying histidine (entries 11, 13 Table 1).

Amino acids carrying alcohol groups were protected with Trt (Thr) and chlorotrityl (ClTrt) (Tyr) groups. Both were removed by 3% TCA (entries 8 and 9, Table 1).

Table 1. Characterization of oligonucleotide 5'-peptide sequences.

No.	oligonucleotide-peptide sequence	side-chain protecting group ^a	HPLC (min) ^b	mass (MALDI) found / calcd	T _m (°C) ^c
1	^N PDP ^C -5'CAATTGG ^{3'}	D(PhiPr)	9.0	2278.3/ 2278.4	--
2	^N PGP ^C -5'CCAATTGG ^{3'}	--	10.4	2838.8/ 2839.7	37.1
3	^N PPLPPGP ^C -5'CCAATTGG ^{3'}	--	11.8	3243.3/ 3244.2	36.5
4	^N PKP ^C -5'CCAATTGG ^{3'}	K(Tfa)	10.7	2908.2/ 2910.8	38.6
5	^N PKEP ^C -5'CCAATTGG ^{3'}	K(MMT) E(PhiPr)	9.9	3036.5/ 3038.9	40.0
6	^N PQ(Trt)P ^C -5'CCAATTGG ^{3'}	Q(Trt)	21.7	3150.3/ 2910.8 ^d	39.6
7	^N PH(Trt)P ^C -5'CCAATTGG ^{3'}	H(Trt)	21.6	3160.5/ 2919.8 ^d	40.2
8	^N PMTP ^C -5'CCAATTGG ^{3'}	T(Trt)	12.2	3013.1/ 3014.9	37.7
9	^N PYQP ^C -5'CCAATTGG ^{3'}	Y(ClTrt)	11.8	3073.3/ 3074.0	44.1
10	^N PHP ^C -5'CCAATTGG ^{3'}	H(Boc)	10.6	2918.3/ 2919.8	39.0
11	^N PHP ^C -5'CCAATTGG ^{3'}	H(Fmoc)	10.7	2917.8/ 2919.8	40.0
12	^N PWP ^C -5'CCAATTGG ^{3'}	--	13.9	2967.5/ 2968.9	43.9
13	^N PHCP ^C -5'CCAATTGG ^{3'}	H(Tos) C(MMT)	11.6	3020.6/ 3022.8	40.1
14	^N AKKKKLDP ^C -5'CCAATTGG ^{3'}	K(Tfa) D(PhiPr)	10.2	3495.2/ 3495	38.5
15	^N CCAATTGG ^{3'}	--	8.8	--	36.5

^aAbbreviations: PhiPr: 2-phenylisopropyl ester, Trt: trityl, Tfa: trifluoroacetyl, MMT: monomethoxytrityl, Boc: t-butyloxycarbonyl, Tos: Tosyl, Fmoc: 9-fluorenyloxycarbonyl, ClTrt: 2-chlorotrityl. ^bRetention time (min) in HPLC (see Supporting Information). ^c1M NaCl, 0.1M sodium phosphate pH 7.0. ^dMass calculated for the trityl-protected derivative.

Fmoc-Trp and Fmoc-Met were successfully used without protection (entries 8 and 12, Table 1). It is important to notice that Met needs protection as sulfone when the peptide is assembled before the oligonucleotide, because it is unstable during the phosphite-to-phosphate oxidation.¹³ In our case the peptide is assembled after the oligonucleotide, so there is no need to protect Met because there is no oxidation step. Unprotected Fmoc-arginine was also tested using carbodiimide activation with an excess of 1-hydroxybenzotriazole to keep the guanidine group of arginine in the protonated form.^{7d} However, peptide elongation did not occur in these conditions. Carbodiimide activation is inefficient within CPG supports.^{7g} Although we did not test it, arginine may be obtained by introduction of the guanidine group in ornithine.¹⁴ Cysteine was protected by the acid labile MMT group (entry 13, Table 1). This conjugate was highly sensitive to ammonia and it was obtained in low yields (see below).

During the synthesis of the PGP-octanucleotide conjugate we used the standard ammonia deprotection conditions (55 °C, overnight). These conditions were also used for the remaining sequences because the amide linkage between peptide and oligonucleotide is stable to ammonia. But when trifunctional amino acids such as histidine or cysteine were present in the peptide sequence the oligonucleotide part underwent severe hydrolysis, as described elsewhere.⁷ This problem was averted by reducing the time of the ammonia treatment (1 h, at 55 °C) or lowering the temperature (overnight, at room temperature).

Finally, some authors have recommended the use of the sarcosyl linkage between oligonucleotide and solid support to avoid premature removal of the oligonucleotide during the piperidine treatment to eliminate the Fmoc groups. In order to assess the extent of this side reaction, we treated oligonucleotide supports with the piperidine solution, followed by analysis of the filtrates. We conclude that this side reaction is negligible in the synthesis of small peptides on oligonucleotide-supports (see supporting information).

Melting temperatures of self-complementary oligonucleotide-peptide conjugates are shown in Table 1. Melting curves were obtained in high salt conditions in order to be able to measure melting temperatures. In agreement with previous reports¹⁵ the presence of the peptide induced a small duplex-stabilizing effect, especially when tryptophan and tyrosine residues are present. Coulombic charge screening by high salt eliminates the beneficial effect of cationic side chains. For this reason peptides carrying Lys and His do not stabilize duplex structure in the conditions used in this work. On the contrary, high salt may push aromatic amino acids (Trp, Tyr) into the minor groove, thus stabilizing the duplex.

In summary, we have developed an efficient and general method for the synthesis of short oligonucleotide-5'-peptide conjugates using commercially available products such as standard DNA synthesis reagents, amino modifiers and Fmoc-protected amino acids. The method allows the preparation of the oligonucleotide-peptide conjugates in any laboratory without the need to prepare special reagents. We believe that this method may have a strong impact on a large number of laboratories by opening the possibility of finding more and more applications for molecules of this type.

Acknowledgment. This study was supported by CICYT (BFU-2004-02048), Generalitat de Catalunya (2001/SGR/0049) and Fundació La Caixa (BM04-52-0). We thank Dr. Maria Macías, Ximena Ramírez, and Dr. Beatriz G. de la Torre for their contribution.

(1) Manoharan, M. *Antisense Nucleic Acid Drug Dev.* 2002, 12, 103-128.

(2) Gait, M.J. *Cell. Mol. Life Sci.* 2003, 60, 844-853.

- (3) Robles, J.; Maseda, M.; Beltrán, M.; Concernau, M.; Pedroso, E.; Grandas, A. *Bioconjug. Chem.* 1997, 8, 785-788.
- (4) a) Tung, C.H.; Stein, S. *Bioconjug. Chem.* 2000, 11, 605-618. b) Eritja R. in *Solid-Phase Synthesis. A Practical Guide.* (Eds.: S.A. Kates, F. Albericio), Marcel Dekker, Inc., New York, 2000, pp. 529-548.
- (5) Corey, D.R. *J. Am. Chem. Soc.* 1995, 117, 9373-9374.
- (6) a) Eritja, R.; Pons, A.; Escarceller, M.; Giralt, E.; Albericio, F. *Tetrahedron* 1990 47, 4113-4120. b) Arar, K.; Monsigny, M.; Mayer, R. *Tetrahedron Lett.* 1993, 34, 8087-8090. c) Ede, N.J.; Tregear, G.W.; Haralambidis, J. *Bioconjug. Chem.* 1994, 5, 373-378. d) Stetsenko, D.A.; Gait, M.J. *J. Org. Chem.* 2000, 65, 4900-49008. e) Antopolsky, M.; Azhayeva, E.; Tengvall, U.; Auriola, S.; Jääskeläinen, I.; Rönkkö, S.; Honkakoski, P.; Urtti, A.; Lönnberg, H.; Azhayev, A. *Bioconjug. Chem.* 1999, 10, 598-606.
- (7) a) de la Torre, B.G., Aviñó, A., Tarrasón, G., Piulats, J., Albericio, F., Eritja, R., *Tetrahedron Lett.* 1994, 35, 2733-2736. b) Soukchareun, S.; Tregear, G.W.; Haralambidis, J. *Bioconjug. Chem.* 1995, 6, 43-53. c) Robles, J.; Beltrán, M.; Marchán, V.; Pérez, Y.; Travesset, I.; Pedroso, E.; Grandas, A. *Tetrahedron*, 1999, 55, 13251-13264. d) Debéthune, L.; Marchán, V.; Fàbregas, G.; Pedroso, E.; Grandas, A. *Tetrahedron*, 2002, 58, 6965-6978. e) Grandas, A.; Marchán, V.; Debéthune, L., Pedroso, E. *Current Protocols in Nucleic Acid Chemistry* John Wiley & Sons, New York, 2004; Chapter 4.22.1. f) Juby, C.D. Richardson, C.D.; Brousseau, R. *Tetrahedron Lett.* 1991, 32, 879-882. g) Truffert, J.C.; Lorthioir, O., Asseline, U., Thuong, .N.T.; Brack, A. *Tetrahedron Lett.* 1994, 35, 2353-2356. h) Bergmann, F.; Bannwarth, W. *Tetrahedron Lett.* 1995, 36, 1839-1842.
- (8) Zanta, M.A.; Belguise-Valladier, P.; Behr, J.P. *Proc. Natl. Acad. Sci. USA* 1999, 96, 91-96.
- (9) Zaramella, S.; Yeheskiely, E.; Strömberg, R. *J. Am. Chem. Soc.* 2004, 126, 14029-14035.
- (10) a) Will, D.W.; Breipohl, G.; Langner, D.; Knolle, G.; Uhlmann, E. *Tetrahedron* 1995, 51, 12069-12082; b) Uhlmann, E.; Peyman, A.; Breipohl, G.; Will, D.W. *Angew. Chem., Int. Ed.* 1998, 37, 2796-2823.
- (11) de la Torre, B.G.; Marcos, M.A.; Eritja, R.; Albericio, F. *Lett. Pept. Sci.* 2002, 8, 331-338.
- (12) De Napoli, L. ; Messere, A. ; Montesarchio, D. ; Piccialli, G. ; Benedetti, E. ; Bucci, E. ; Rossi, F. *Biorg. Med. Chem.* 1999, 7, 395-400.
- (13) Marchán, V.; Rodríguez-Tanty, C.; Estrada, M.; Pedroso, E.; Grandas A. *Eur. J. Org. Chem.* 2000, 2495-2500.
- (14) a) Antopolsky, M.; Azhayev, A. *Tetrahedron Lett.* 2000, 41, 9113-9117. b) Antopolsky, M.; Azhayeva, E.; Tengvall, U.; Azhayev, A. *Tetrahedron Lett.* 2002, 43, 527-530.
- (15) Marchán, V.; Debéthune, L., Pedroso, E.; Grandas, A. *Tetrahedron* 2004, 60, 5461-5469.

Supporting Information

A straightforward synthesis of 5'-peptide oligonucleotide conjugates using N^α-Fmoc- protected amino acids.

Sandra M. Ocampo¹, Fernando Albericio^{*,2,3}, Irene Fernández³, Marta Vilaseca³ and Ramon Eritja^{*,1,2}

¹Institut de Biologia Molecular de Barcelona. C.S.I.C., Jordi Girona 18-26, E-08034 Barcelona, Spain.

²Institut de Recerca Biomédica de Barcelona, Parc Científic de Barcelona, Josep Samitier 1-5, E-08028 Barcelona. Spain.

³Departament de Química Orgànica, Facultat de Química. Universitat de Barcelona, Martí i Franqués 1-11. E-08028 Barcelona. Spain.

Abbreviations: DCM: dichloromethane; CPG: controlled pore glass; DIPEA: diisopropylethylamine; DMF: N,N-dimethylformamide; Fmoc: (9-fluorenyl)methoxy)carbonyl; LV200: polystyrene support (low volume); MMT: monomethoxytrityl; PyBOP: Benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate; O.D. optical units; TEAA: triethylammonium acetate; TCA trichloroacetic acid.

Experimental Section.

Synthesis of oligonucleotide-peptide conjugates.

Oligonucleotides were prepared on an automatic Applied Biosystems 392 DNA synthesizer on several batches of 1 μmol scale (CPG) or 0.2 μmol scale (polystyrene, LV200) using commercially available reagents (Applied Biosystems, USA) following standard protocols. The phosphoramidite of MMT-6-aminohexanol derivative (Cruachem, Scotland and Glen Research, USA) was used for the incorporation of an amino group at the 5'-end of the sequence. During the addition of the amino linker we avoided the use of the capping step to prevent partial acetylation of the amino group. The MMT group was removed using 3% TCA in DCM and the resulting support was stored in the refrigerator in the protonated form.

Solid-phase peptide synthesis were performed manually in a batch procedure using N^α-Fmoc-protected amino acids (Bachem, Germany and Novabiochem, Switzerland). Syntheses were performed on 0.2-0.5 μmol scale. The following synthesis protocol was

applied for all conjugates. First, the support was neutralized with 5% DIPEA in DCM and washed in CH₃CN. The resulting support was treated with a mixture of Fmoc-amino acid (20 equiv), PyBOP (20 equiv) and DIPEA (40 equiv) in 0.2-0.3 ml of DMF for 30 min. Then, the solution was filtered and the support was washed in DMF and CH₃CN.

The Fmoc group was then removed using a 20% piperidine solution in DMF for 5 min and the support was washed in CH₃CN. At the end of the synthesis if acid-labile groups were used for the protection of the side chains of the amino acid, the support was treated with a 3% TCA solution in DCM for 10 min and washed in CH₃CN. After the assembly of the sequences, supports were treated with 32% aqueous ammonia at 55 °C for 16 h. In some cases, when trifunctional amino acids (such as histidine, cysteine) were present the oligonucleotide moiety underwent severe hydrolysis, which was strongly reduced by treating the supports with 32% aqueous ammonia at 55 °C for 1 h or at room temperature overnight.

Ammonia solutions were concentrated to dryness and the products were desalted over a Sephadex G-25 (NAP-25) column eluted with water. Yields 7-15 O.D. units at 260 nm. The resulting products were further purified by reversed-phase HPLC. Solutions were as follows. Solvent A: 5% CH₃CN in 100 mM TEAA pH 6.5 and solvent B: 70% CH₃CN in 100 mM TEAA pH 6.5. Columns: PRP-1 (Hamilton), 250 x 10 mm. Flow rate: 3 ml/min. A 20 min linear gradient from 0-50% B and 5 min isocratic at 50% B. Yields 2-6 O.D. units at 260 nm, except for the conjugate PHCP-octamer that was 0.6 O.D. units. The HPLC retention time, mass spectrometry and melting temperature data are shown in Table 1.

Carboxylic acid versus carboxamide during ammonia deprotection of 2-phenylisopropyl esters.

In order to check whether the PhiPr group was completely removed with the TCA treatment we used a model tripeptide (PheGluPro, FEP). First FEP-amide and FQP-amide were prepared using standard Fmoc-chemistry on polystyrene (Rink amide resin, Novabiochem, Switzerland). FEP-amide, MS (MALDI-TOF): Calcd. 391.4; found 413.23 (M+ Na) and 429.21 (M+K), HPLC (see conditions above) 10.0 min; FQP-amide, MS (MALDI-TOF): Calcd. 390.4; found 413.22 (M+ Na) and 429.18 (M+K), HPLC (see conditions above) 8.25 min.

Next, two aliquots of PheGlu(PhiPr)Pro-support were treated: (I) with concentrated ammonia (55 °C overnight) and (II) first with 3% TCA for 10 min followed by concentrated ammonia (55 °C overnight). Afterwards, the resulting supports were collected and treated with 95% TFA/ 5% water to remove the peptide from the support. HPLC analysis (see conditions above) of the resulting products indicated that the direct treatment of PheGlu(PhiPr)Pro-support with ammonia produced a mixture of two tripeptides: 15% of the tripeptide with glutamine and 85% of the peptide with glutamic acid. The same tripeptide-support treated with ammonia after a 10 min treatment with 3% TCA in DCM yielded the tripeptide carrying glutamic acid as single compound (>95%). This indicates that the PhiPr group was completely removed by 3% TCA solution followed by ammonia yielding the desired oligonucleotide-peptide conjugates.

Stability of succinyl linkage to piperidine.

A 5 mg aliquot of DMT-^{5'}CCAATTGG^{3'}-succinyl-CPG support was treated with 20% piperidine/ DMF for 1 h at room temperature. The filtrate was concentrated to dryness and the resulting residue was first dissolved in water and then analysed by UV-spectroscopy at 260 nm. The aqueous solution was concentrated to dryness and 1 ml of perchloric acid/ ethanol (3:2) was added. The absorption of the solution at 500 nm was measured. Next, the support obtained after the piperidine treatment was treated with 3% TCA/ DCM and the coloured DMT cation solution was collected and dried, and 1 ml of perchloric acid/ ethanol (3:2) was added to the resulting residue. The remaining support was treated with concentrated ammonia (1 h, 55 °C) and filtered. The filtrates were analysed by UV-spectroscopy at 260 nm. Comparison of the DMT cation and DNA absorbance between filtrates and support after piperidine treatment gave a loss of 5-10% of DMT-oligonucleotide after one hour of treatment. This result is consistent with a study of the stability of oligonucleotides linked to solid supports to bases (Aviñó, A., Güimil García, R., Díaz, A., Albericio, F., Eritja, R. *Nucleosides Nucleotides* **1996**, *15*, 1871-1889) and shows that premature removal oligonucleotide from succinyl supports during piperidine treatments needed for the removal of the Fmoc groups (5 min per cycle) is about 0.4-0.8% per cycle. We conclude that this side reaction is negligible in the synthesis of small peptides on oligonucleotide-supports.

Melting experiments. Melting experiments were performed as follows. Solutions of the appropriate oligonucleotide-peptide conjugates (approx. 0.5 O.D. units) were prepared in a solution containing 1M NaCl, 100mM sodium phosphate buffer of pH 7.0. The solutions were heated to 90 °C, allowed to cool slowly to room temperature and stored at 4 °C until UV measurement. UV absorption spectra and melting experiments

(absorbance vs temperature) were recorded in 1 cm path-length cells using a spectrophotometer, with a temperature controller and a programmed temperature increase rate of 0.5 °C/min. Melting curves were recorded at 260 nm and melting temperatures were measured at the maximum of the first derivatives of the melting curves.

Figure S1: HPLC trace of crude N Pro-Gly-Pro C -5'CCAATTGG 3 prepared on polystyrene supports (LV200) using capping after the addition of each amino acid (see conditions in experimental section). The major peak at 10.3 min shows the expected molecular weight for the desired oligonucleotide-peptide conjugate. Other side products observed in the mass spectra of the crude are octanucleotide (failure on the addition of the 5' amino modifier) and 5' acetyl-amino-octanucleotide (acetylation of 5'-amino oligonucleotide)

Figure S2: HPLC trace of crude ${}^N\text{Pro-His-Pro}^{\text{C-5'}}\text{CCAATTGG}^{\text{3'}}$ prepared on controlled-pore glass (CPG) using the tert-butoxycarbonyl group (Boc) for the protection of histidine (see conditions in experimental section). Ammonia treatment was performed at 55 °C for 1 h. The major peak at 10.6 min shows the expected molecular weight for the desired oligonucleotide-peptide conjugate.